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COMPARATIVE BIOCHEMICAL ASSESSMENT OF THE COTTON BACTERIAL BLIGHT PATHOGEN

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Abstract

Bacterial blight, caused by Xanthomonas citri pv. malvacearum (Xcm), is a significant disease impacting cotton production in Pakistan. Effective disease management relies on a comprehensive understanding of the pathogen's characteristics. In this study, Xcm was isolated from infected cotton samples collected in the Bahawalpur region and subsequently purified. Identification was carried out using morphological, biochemical, and cultural analyses.

Biochemical testing indicated that the isolates were positive for potassium hydroxide (KOH), catalase, oxidative-fermentative (OF) test, urease, starch hydrolysis, citrate utilization, methyl red, Voges-Proskauer, and casein hydrolysis, while they were negative for Gram staining, indole production, and arginine dihydrolase activity. Cultural characterization revealed mucoid, convex colonies with yellow to orange pigmentation on nutrient agar, and flattened or slightly raised mucoid colonies ranging from bright to pale yellow on yeast extract dextrose calcium carbonate (YDCA) agar.

These findings provide critical insights for researchers, aiding in the accurate identification of Xcm and supporting in vitro evaluation of potential strategies to control bacterial blight in cotton.

INTRODUCTION

Cotton (Gossipium hirsutum L.) belongs to the Malvaceae family and is known as the backbone of the economy of Pakistan by contributing 0.6% to GDP and 2.4% to total value addition in agriculture (Sahu et al., 2022). Pakistan is the 5th largest producer of cotton in the world, after Brazil, India, China, and the United States (Khalid et al., 2022). During 2020–21, Pakistan contributed 5.0% of global cotton production. Pakistan holds the position of the second-largest global yarn exporter, ranks third in terms of cotton consumption, and is the second-largest exporter of textile products (Akhtar et al., 2024). Cotton productivity faces challenges from various biotic and abiotic factors, including several diseases that threaten cotton yield. Among these diseases, bacterial blight is the most severe, caused by Xanthomonas citri pv. malvacearum (Xcm) (Manna et al., 2024). This bacterium falls within the Xanthomonadaceae family, which stands as the most extensive and significant group of plant pathogens. X. citri pv. malvacearum enters into the host plant through the help of natural openings such as stoma and hydathode or wound. Wind-driven rain splashes help disseminate it to different areas of the plant (Naqvi et al., 2022). Once it gains entry, it proliferates within the intracellular spaces, contributing to the development of disease (Aarrouf et al., 2008). X. citri pv. malvacearum is capable of surviving in plant debris for about over a year. Under favorable weather conditions, yield losses of cotton seed can exceed 50%. In cotton fields affected by

bacterial blight, yield reductions of up to 80% have been observed (Naqvi et al., 2022).

In the investigation of pathogenesis, evolutionary relationships, and the interaction between the bacterium and its host X. citri pv. malvacearum is used as a model organisms (Netrusov et al., 2023). X. citri pv. malvacearum uses specific proteins which help in adhesins, that play a role in the establishment of the bacterium within the host. After adhering, the bacterium uses the secretion system of type III to transfer its effector proteins inside the plant. These effector proteins alter the plant defence providing benefit to the pathogen. Afterward, the formation of biofilms contributes to enhancing the bacterial population by improving adhesion, with adhesins and the exopolysaccharide xanthan playing a crucial role in this process (Brunings & Gabriel, 2003). The bacterium adheres to the surface of cotton leaves leading to the development of symptoms in susceptible plants (Turechek et al., 2023).

Known as the most destructive bacterial disease affecting cotton plants, it targets the host throughout all stages of plant growth, infecting seedlings, bolls, stems, and leaves (Jagtap et al., 2012). This results in the blight of cotton seedlings, angular leaf spots, black arms, and lesions on the boll. These symptoms manifest as dark green in color, water- soaked, angular-shaped lesions ranging in size from 1 to 5 mm, particularly noticeable on the underside of leaves. Occasionally, there are extensive dark green lesions with a water-soaked appearance along the veins. Typically, the prevalence of these symptoms is high on lower leaves compared to upper leaves of cotton. Over time, the lesions dry up and darken, and the affected leaves tend to fall prematurely, ultimately resulting in extensive defoliation (Mijatović et al., 2021).

Different strains of Xanthomonas have been recognized and differentiated through a range of techniques. Various biochemical and cultural tests, including gram staining, starch hydrolysis, potassium hydroxide (KOH), citrate utilization, catalase, oxidative fermentative (OF), methyl red, urease, Voges-Proskauer, casein hydrolysis, indole production, arginine di-hydrolase, gelatin liquefaction testing, as well as growth of bacterial colony on nutrient agar (NA) and yeast extract dextrose calcium carbonate agar (YDCA) media. These tests serve as markers for identification, aiding in the bacterial identification process (Reynolds et al., 2005). Biochemical and cultural analysis of the bacterium holds significant importance in disease control efforts. Additionally, it plays a crucial role in monitoring the virulence of pathogens and screening susceptible cotton varieties. The current study aimed to isolate, identify, and comprehensively profile of Xcm strain affecting cotton in Bahawalpur, Pakistan, taking into consideration the aforementioned context.

1. Materials and Methods

1.1. Sample collection

Cotton leaves exhibiting typical symptoms of bacterial blight were collected from a cotton field in the Bahawalpur region, Punjab Pakistan (29.3981° N, 71.6908° E). These affected leaves were carefully placed into plastic envelopes and transported to the Plant Pathology Laboratory, Department of Plant Pathology at The Islamia University of Bahawalpur, Punjab Pakistan for subsequent analysis.

1.2. Isolation and purification of Xanthomonas citri pv. malvacearum

Infected leaf samples were cut into 2 × 2 mm discs and surface-sterilized with 70% ethanol, followed by rinsing with autoclaved distilled water. General bacterium growth medium (NA) was prepared by dissolving peptone, beef extract, and agar 5 g, 3 g, and 15 g respectively in 1,000 ml of distilled water. Leaf discs were ground in sterilized distilled water using a pestle and mortar. Small portions of suspension were streaked onto petri plates containing nutrient agar with a sterilized loop and incubated at 28 ± 2°C for 24 hours. The resulting colonies were sub-cultured on fresh plates of nutrient agar under the same conditions. Three purified cultures (Xcm1, Xcm2, Xcm3) were stored at 4°C for further use remaining viable for 4–6 weeks (Jadhav et al., 2018).

1.3. Pathogenicity test In-Vitro condition.

To confirm the pathogenicity of Xcm, a detached leaf assay was conducted. The process involved surface disinfection of the leaves using 50% ethanol, followed by rinsing them with distilled water.

Subsequently, the leaves were positioned on 1% water agar medium. Wounds were carefully created on leaves using a sterilized needle, and on each wound, a bacterial suspension containing 10^6 CFU/ml was applied. This suspension was prepared using a serial dilution method. The treated leaves were then placed in an incubator at 28±2℃ for a duration of 1 week. As a negative control, sterile water was used (Burlakoti et al., 2018).

1.5. Biochemical characterization of Xanthomonas citri pv. malvacearum

Bacteria that cause diseases in plants display a diversity of biochemical and cellular activities, which can vary significantly from one pathogen to another. To distinguish and understand the specific biochemical characteristics of these bacteria, various biochemical tests have been developed.

1.5.1. Gram staining

A bacterial smear was mixed on a microscope slide by homogenizing a pure culture of Xcm with a few drops of sterile water. Firstly, for staining the smear was subjected to crystal violet for a duration of 30 seconds, then rinsed with distilled water to eliminate extra crystal violet. Secondly, a few iodine drops were applied to the bacterial culture for 30 seconds and subsequently rinsed by distilled water. Thirdly, a few drops of ethanol were applied for 15 seconds, followed by another rinse with distilled water. Finally, a few drops of safranin were applied for counterstaining and allowed to settle for 30 seconds before washing the slide with distilled water. The slide with the bacterial colony was then dried using filter paper and examined under a light microscope. Gram-positive bacteria would retain the stain of crystal violet appearing violet or purple in color. On the other hand, Gram- negative bacteria would lose the stain of crystal violet and appear in pink color (Razaghi et al., 2012).

1.5.2. Potassium hydroxide (KOH) Test

On a clean glass slide, several drops of 3% (w/v) potassium hydroxide were added, and a 48-hour-old bacterial culture was mixed with a sterile inoculation loop, followed by a 10-second stirring. The presence of sticky thread visible when lifting the wire loop indicated the Gram-negative nature of the bacteria (Khan et al., 2024).

1.4. Cultural characteristics of Xanthomonas citri pv. malvacearum

The characteristics related to the culture, including colony shape, border, height, surface, and coloration, of three different test isolates were investigated through the use of nutrient agar (Somashetty et al., 2023).

1.5.3. Catalase test

A sterile loop containing the culture of the tested bacteria aged between 24 to 48 hours was placed onto a glass slide. Subsequently, a few drops of H_2O_2 were added onto the slide and allowed the mixture to react for a brief period. The appearance of gas bubbles showed a catalase-positive bacteria and vice versa (Khan et al., 2024).

1.5.4. Oxidative fermentative (OF) test

A basal medium was formulated for the OF test, which included 5 grams of sodium chloride, 3 grams of di- potassium phosphate, 2 grams of peptone, 0.03 grams of bromothymol blue, 3 grams of agar, and dissolved in 1000ml of distilled water. The pH of the medium was adjusted to 7.1 before subjecting it to autoclaving. After autoclaving, a sterile solution of 10% Glucose was filter-sterilized and added to the medium aseptically to attain a required concentration of 1%. For each bacterial isolate, two test tubes were inoculated, with one of them being covered with sterile liquid paraffin. A color change from blue to yellow in both tubes indicates positive anaerobic growth (Li et al., 2024).

1.5.5. Urease test

A urease medium was prepared by mixing 1 gram of dextrose, 1.5 grams of peptone, 5 grams of sodium chloride, 2 grams of monopotassium phosphate, 0.012 grams of phenol red, and 15 grams of agar, and then adding deionized water. The pH of the medium was adjusted to 6.7, and the medium was autoclaved at 121°C for 20 minutes, except for the urea component. After autoclaving, the medium was cooled to a temperature between 50 to 55°C, and then the urea base was thoroughly mixed into the medium. Tubes were tilted during cooling until they solidified. The isolates were introduced onto the

slant surface and subjected to incubation at a temperature of 28±2°C for a period of 48 hours (Sayekti et al., 2024).

1.5.6. Starch hydrolysis

A starch agar medium was prepared, comprising 5 grams of peptone, 3 grams of beef extract, 2 grams of starch, and 15 grams of agar dissolved in 1000ml of distilled water. This mixture was then autoclaved. Bacterial culture was streaked onto the plates and incubated for 24 hours until growth became visible. After incubation, Lugol's iodine was applied to the plates and allowed to react briefly. A clear, yellowish area surrounding the bacterial growth indicated a positive result, while the absence of such an area signified a negative results (Van Phuong et al., 2021).

1.5.7. Citrate test

Simmons's citrate agar, dissolved 5 grams of sodium chloride, 2 grams of sodium citrate, 1 gram of ammonium dihydrogen phosphate, 1gram of dipotassium phosphate, 0.2 gram of magnesium sulfate, 0.08 grams of bromothymol blue, and 15 grams of agar in 1000 ml of distilled water and adjusted to pH 6.7. This medium was autoclaved at 121°C for 20 minutes. After autoclaving, the medium was poured into Petri dishes, after solidifying. Bacterial isolates were streaked onto agar plates and incubated at 28 ± 2°C for 24 hours. A color change from green to bright blue was considered a positive result, while no change in the color of the medium indicated a negative result (Patidar & Ranjan, 2022).

1.5.8. **Casein hydrolysis test**

Skim milk agar medium was prepared by combining 28 grams of skim milk powder, 5 grams of casein enzymic hydrolysate, 2.5 grams of yeast extract, 1 gram of D-glucose, and 15 grams of agar dissolved in 1000 ml of distilled water and autoclaved it. The bacterial culture was then streaked onto the media and incubated for 24 hours. A positive test was shown by the existence of a distinct zone encircling the bacterial growth, while a negative test was shown by the absence of a zone (Chatterjee, 2023).

1.5.9. **Methyl red test**

Media was prepared by dissolving 7 grams of peptone, 5 grams of dextrose, and 5 grams of dipotassium phosphate in 1000ml of distilled water. This medium was sterilized. The bacterial culture was inoculated onto the medium and allowed to incubate for 48 hours. Following incubation, an indicator, methyl red was added. Any changes in color observed were indicative of a positive reaction (Naik et al., 2018).

1.5.10. **Voges-Proskauer (VP) test**

A test tube containing VP medium was prepared by dissolving 7 grams of peptone, 5 grams of dextrose, and 5 grams of dipotassium phosphate in 1000 ml of distilled water. This medium was sterilized. The medium was inoculated with the bacterial culture and allowed to incubate for 48 hours. After this incubation period, VP-I (5% alpha- naphthol) and VP-II (40% potassium hydroxide) reagents were added, and any color changes observed after a 2-hour interval show a positive results (Kure et al., 2022).

1.5.11. **Indole production test**

A Tryptophan broth medium was prepared by dissolving 10 grams of casein enzymic hydrolysate, 5 grams of sodium chloride, and 1 gram of tryptophan in 1000 ml of distilled water and the medium was sterilized. The bacterial culture was then inoculated into the test tube and incubated for 24 hours at a temperature of 28±2°C. After incubation, 0.3ml of Kovac's reagent was added. A positive result was confirmed by the presence of a pink-colored ring formation, while a negative result was indicated by the absence of any color change (Sarker et al., 2017).

1.5.12. **Arginine Di-hydrolase test**

The media was prepared by using 1 gram of peptone, 5 grams of sodium chloride, 0.3 grams of dipotassium hydrogen phosphate, 3 grams of agar, 0.01gram of phenol red, and 10 grams of L-arginine monohydrochloride, all mixed in 1000ml of distilled water and adjusted the pH 7.2. This medium was sterilized through autoclaving at 121°C for 20 minutes and was then distributed in 5 ml into test tubes. The medium was inoculated with bacteria, and the surface was covered with a 5 mm layer of

sterile liquid paraffin. After overnight incubation, a positive test was indicated by the entire medium turning pink in color. Negative tests remained unchanged or exhibited a yellow coloration, signifying acid production (Nauman et al., 2023).

1.5.13. **Gelatin liquefaction test**

For the Gelatin Liquefaction test, fresh gelatin medium was prepared by dissolving 3 grams of beef extract, 5 grams of peptone, and 120 grams of gelatin in distilled water making the final volume of 1000mL. 5 ml of prepared medium was poured into each test tube. Tubes containing media were plugged and sterilized for 20 minutes at 121°C and cooled until inoculated. Media in tubes were stab inoculated with cells from 24-hour agar slants and were incubated at 28 ±2°C. After 3, 7, 14, and 21 days, tubes were placed at 4°C for 30 minutes in an ice water bath prior to recording the results. In the case in which gelatin was hydrolyzed, the medium flowed readily as the tube was gently tipped. In the case of unhydrolyzed gelatin, the medium was unable to flow (Naik et al., 2018).

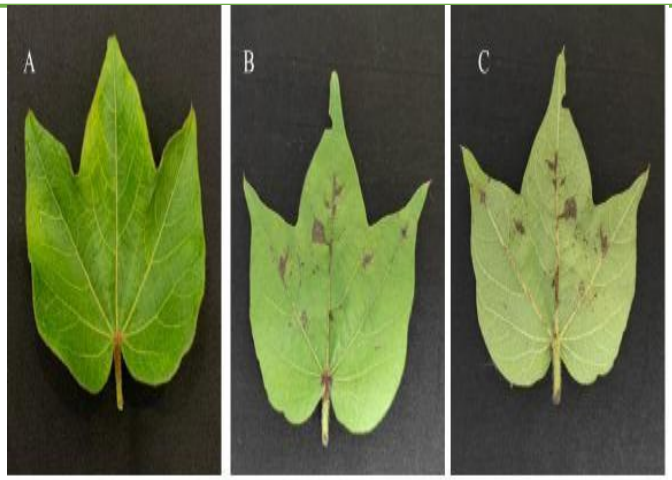
2. Results

2.1. Sample collection

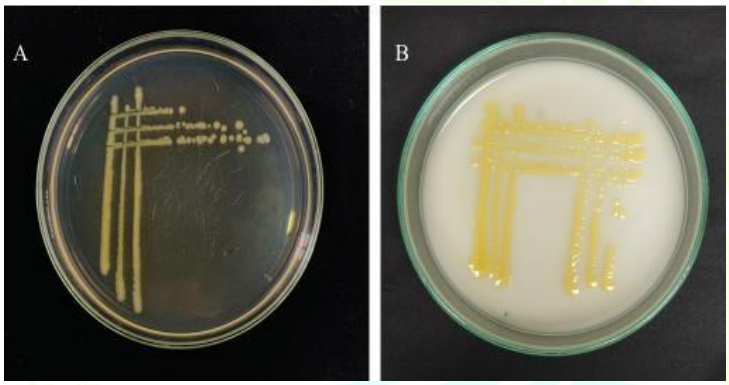
From 2022 to 2023, bacterial blight of cotton was commonly observed in Bahawalpur, Punjab, Pakistan. Symptoms were observed as angular leaf spots, necrotic lesions, defoliation, and black shown in Figure. 1.

2.2. Pathogenicity test

The pathogenicity test was conducted using the pinprick technique on healthy cotton leaves. After an incubation period of one week, symptoms appeared in the form water- soaked lesion on the leaf, subsequently turning yellow as it expanded in both length and width, forming an elongated and irregular lesion shown in Figure. 2. Xanthomonas citri pv. malvacearum was reisolated from the inoculated leaves and its identification was confirmed through the examination of its phenotypic characteristics.



2.3. Cultural characteristics



On NA medium, colonies exhibited a convex shape and showed a mucoid texture with colors ranging from yellow to orange. However, on YDCA medium colonies appeared viscous, slightly elevated, and had a pale yellow, mucoid appearance as shown in Figure 3.

2.4. Gram staining

Microscopic analysis of a Gram-stained sample of X. citri pv. malvacearum revealed that the examined bacteria were gram-negative and had a straight rod shape.

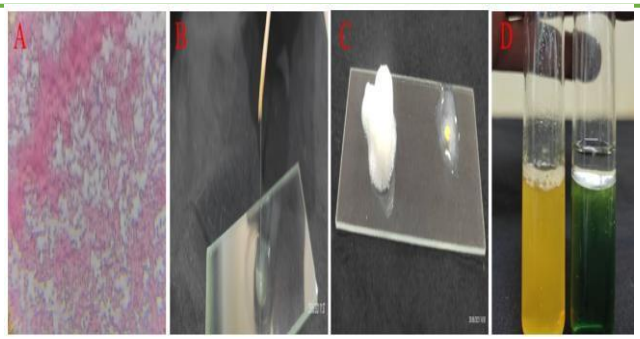
2.5. Potassium hydroxide (KOH) test

The presence of slimy threads or loops is an indicator of the bacteria being gram-negative. In the present study, the tested bacterium displayed a positive response in the KOH test.

2.6. Catalase test

The tested bacteria were mixed with a few drops of H_2O_2 on a clean glass slide, which produced bubbles of gas, indicating a positive catalase test.

2.7. Oxidative fermentative (OF) test



In the tubes without paraffin oil, a yellow color was observed, while in the tubes containing paraffin oil, no color change occurred. This test served to confirm the true aerobic nature of the bacteria.

2.8. Urease test

The isolated bacteria showed a negative result in the urease test, indicating that the tested bacteria did not possess the ability to catalyze urea.

2.9. Starch hydrolysis

When the tested bacteria were cultivated on a medium flooded with Lugol's iodine, the starch hydrolysis test showed a positive result for the test.

2.10. Citrate utilization test

After two days, all of the isolates showed a positive result for citrate utilization, indicating that they can use citrate as a carbon source.

2.11. Methyl red

When the addition of the methyl red reagent causes a color change from yellow to red after 24 hours of incubation, the result is positive.

2.12. Voges-Proskauer test

In this test, all the isolates exhibited positive results, confirming their capacity for acetoin production.

2.13. Casein hydrolysis test

All the tested isolates showed positive results, indicating the presence of the required proteolytic exoenzyme for casein hydrolysis.

2.14. Indole production test

After the incubation of cultured isolate on tryptone broth, Kovac's reagent was added and all the isolates showed negative results.

2.15. Arginine Di-hydrolase test

The isolates that were inoculated showed an inability to utilize arginine. During the test, no color change was observed, and all the isolates tested as negative.

2.16. Gelatin liquefaction

All Xcm isolates displayed the ability to transform the gelatin medium into a liquid condition as compared to the control group. All the isolates tested positive.

3. Discussion

This investigation aimed to isolate and validate the presence of X. citri pv. malvacearum collected from various locations within Bahawalpur. Xanthomonas spp, belonging to the genus Xanthomonas, are typically straight, rod, and Gram-negative. They are predominantly yellow-pigmented and often possess a polar flagellum. These bacteria are obligate aerobic chemoorganotrophs, with a significant portion of species being phytopathogens. After 48 hours of incubation at 28±2°C, bacterial colonies became visible on Nutrient Agar plates. A visual examination was conducted to characterize the appearance of these colonies. They exhibited a yellow color and were characterized by their relatively small to medium size, convex shape, and mucoid consistency (Jaskani, 2021).

The microscopic study of Gram-stained sample of X. citri pv. malvacearum revealed that the bacterium under investigation did not retain the Crystal violet stain. Instead, its cells appeared pink, showing safranin had been used as a counter stain. Consequently, it was concluded that the examined bacterium was gram-negative and had a straight

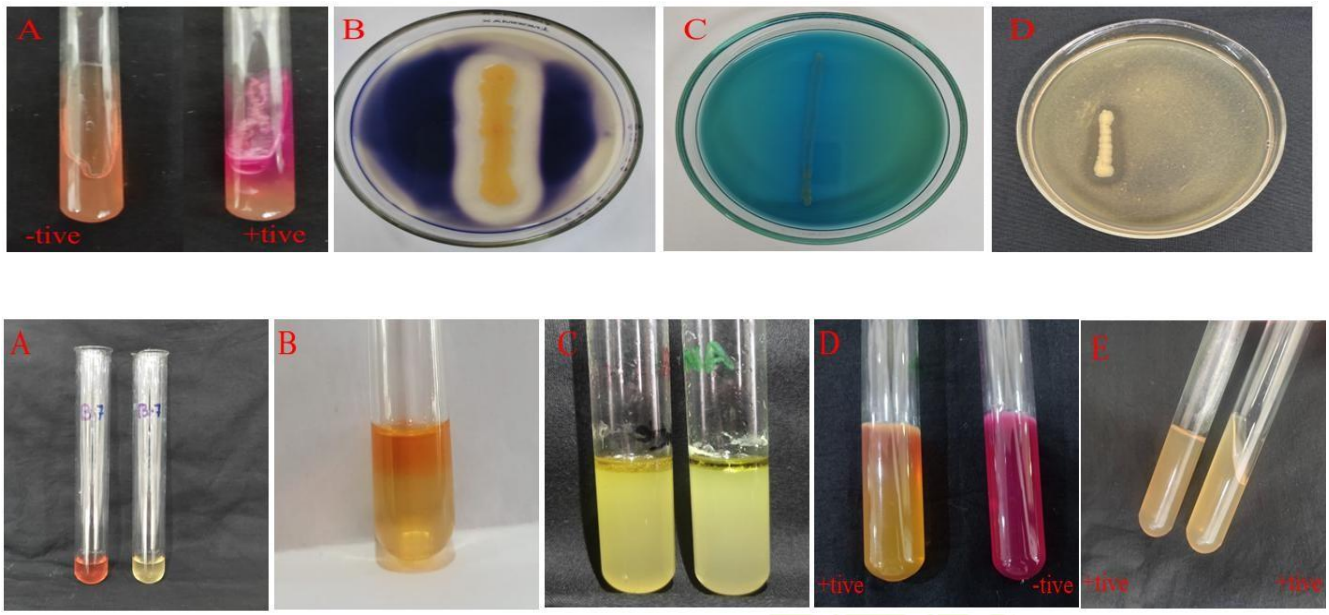


Figure. 5 A) urease test **B)** starch hydrolysis **C)** citrate utilization test, **D)** methyl red

Figure. 6 A) Voges-Proskauer test, **B)** casein hydrolysis test **C)** indole production test, **D)** arginine Di-hydrolase test, **E)**
gelatin liquefaction

Table 1 Biochemical characteristics of X. citri pv. malvacearum

Test	Xcm 1	Xcm 2	Xcm 3
Gram staining	–ve	–ve	–ve
Potassium hydroxide (KOH) test	+ve	+ve	+ve
Catalase test	+ve	+ve	+ve
Oxidative fermentative (OF) test:	+ve	+ve	+ve
Urease test	–ve	–ve	–ve
Starch hydrolysis	+ve	+ve	+ve
Citrate Utilization test	+ve	+ve	+ve
Methyl Red	+ve	+ve	+ve
Voges-Proskauer test	+ve	+ve	+ve
Casein Hydrolysis test	+ve	+ve	+ve
Indole production test	–ve	–ve	–ve
Arginine Di-hydrolase test	–ve	–ve	–ve
Gelatin liquefaction	+ve	+ve	+ve

rod shape characteristic, a distinct trait commonly linked with plant pathogenic bacteria. These results align with the research conducted by Kamble et al., 2019.

The presence of slimy threads or loops implies that the bacterium is gram-negative. This shows that gram-negative bacteria have relatively thin cell walls, surrounded by an outer membrane. The bacteria cell wall is readily destroyed by exposing it to a 3% KOH solution, resulting in the release of DNA. Conversely, gram-positive bacteria possess a denser and stronger cell wall that can resist the disruptive action of KOH. These outcomes are consistent with the earlier research conducted by Jabeen et al, 2012.

The enzyme catalase promotes cellular detoxification by the hydrolysis of H2O2 into oxygen and water. This finding is consistent with the outcomes reported by Mohammadi et al., 2001. However, due to the absence of the urease enzyme, the bacteria did not exhibit urea catalysis capabilities which convert urea into ammonia and carbon dioxide. This result corresponds to the findings reported by Mohammadi et al., 2001.

The occurrence of a colorless zone surrounding the bacterial growth shows that the bacteria use the exoenzyme amylase for the breakdown of starch. This process results in the hydrolysis of starch into dextrins, maltose, and glucose/alpha-amylase. These findings consisted of the research conducted by Jabeen et al., 2012.

Xcm exhibited a positive response in the citrate utilization test. This shows that the bacteria have the ability to use citrate as a sole carbon source and inorganic ammonium salts as the sole nitrogen source. It results in the formation

of citrate permease, which converts citrate into pyruvate. Moreover, pyruvate incorporated with the bacterial metabolic cycle results in generating energy. Ammonium salts break down metabolic processes resulting in enhancing alkalinity. These findings correspond with the study reported by Arshad et al., 2015.

Xcm shows positive results in both the methyl red and Voges-Proskauer tests. It ferments sugars via the butanediol fermentation pathway, which results in limited acidification of the culture medium. During sugar fermentation, the pH indicator, i.e., methyl red, was quite efficient for measuring hydrogen ion concentration. Further, Xcm produces acetoin as an intermediate in the sugar fermentation process, which ultimately reduces to form 2,3-butanediol. These outcomes closely resemble the results reported in the prior research conducted by Ogunjobi et al., 2008.

The Casein Hydrolysis test is used to recognize bacteria that produce proteolytic exoenzymes responsible for the hydrolysis of casein into amino acids. The presence of a clear zone surrounding the bacterial growth showed positive results, indicating effective casein hydrolysis. However, in this specific test, the absence of a transparent area was noticed, demonstrating that the isolated bacteria were unable
to hydrolyze casein. This finding resembles the results documented by Goszczynska & Serfontein (1998).

In the presence of bacteria that produce the tryptophanase enzyme, Tryptophan, an amino acid, undergoes deamination and hydrolysis. Through reductive deamination, the enzymatic process leads to indole formation. Change in the solution color was observed by mixing the indole with Kovacs reagent. These outcomes resemble the study conducted by Arshad et al., 2015.

The isolates that were added to the culture showed the inability to consume arginine as a source of carbon and energy for their growth. This limitation was attributed to the absence of the enzyme arginine dihydrolase, which liberates ammonium from arginine, as confirmed in the study by Raghuwanshi et al., 2013.

This test examines the bacterial ability to produce extracellular proteolytic enzymes, known as gelatinases, which liquefy gelatin. This process possesses two consecutive stages: first, the gelatinases hydrolyze gelatin into polypeptides and these polypeptides are further converted into amino acids. Bacterial cells absorb these amino acids and use them in metabolic activity. These findings align with earlier research conducted by Suryawanshi et al., 2011.

The pathogenicity of purified Xcm was evaluated by using detached cotton leaves. However, it is noted that this study serves as a foundational reference point for the successful management of the Bacterial Blight of Cotton, which currently poses a great threat to cotton yield in Pakistan.

4. Conclusion

The competitiveness of the Xcm isolates examined in this study provides valuable insights for implementing effective management practices in various locations. Morphological and biochemical characterization serves as the foundation for comprehending pathogen populations and biological behaviors, contributing to a comprehensive understanding of pathogen aggressiveness. To gain a complete understanding of pathogenic mechanisms, it is advisable to conduct molecular studies.

5. Funding

No funding was received for this study.

8. Conflict of Interest

The authors declare no conflict of interest.

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